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 - 2) Shimizu, Tatsuya, 2002, Circulation res, 90(3): pe40.
 - 3) Aziz, S, 1997, Transplantation International. Official J of the Eur Soc Organ Transplantation (Germany), 10(6): 446-50.
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Fabrication of Pulsatile Cardiac Tissue Grafts Using a Novel 3-Dimensional Cell Sheet Manipulation Technique and Temperature-Responsive Cell Culture Surfaces

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Abstract—Recent progress in cell transplantation therapy to repair impaired hearts has encouraged further attempts to bioengineer 3-dimensional (3-D) heart tissue from cultured cardiomyocytes. Cardiac tissue engineering is currently pursued utilizing conventional technology to fabricate 3-D biodegradable scaffolds as a temporary extracellular matrix. By contrast, new methods are now described to fabricate pulsatile cardiac grafts using new technology that layers cell sheets 3-dimensionally. We apply novel cell culture surfaces grafted with temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), from which confluent cells detach as a cell sheet simply by reducing temperature without any enzymatic treatments. Neonatal rat cardiomyocyte sheets detached from PIPAAm-grafted surfaces were overlaid to construct cardiac grafts. Layered cell sheets began to pulse simultaneously and morphological communication via connexin43 was established between the sheets. When 4 sheets were layered, engineered constructs were macroscopically observed to pulse spontaneously. In vivo, layered cardiomyocyte sheets were transplanted into subcutaneous tissues of nude rats. Three weeks after transplantation, surface electrograms originating from transplanted grafts were detected and spontaneous beating was macroscopically observed. Histological studies showed characteristic structures of heart tissue and multiple neovascularization within contractile tissues. Constructs transplanted into 3-week-old rats exhibited more cardiomyocyte hypertrophy and less connective tissue than those placed into 8-week-old rats. Long-term survival of pulsatile cardiac grafts was confirmed up to 12 weeks. These results demonstrate that electrically communicative pulsatile 3-D cardiac constructs were achieved both in vitro and in vivo by layering cardiomyocyte sheets. Cardiac tissue engineering based on this technology may prove useful for heart model fabrication and cardiovascular tissue repair. The full text of this article is available at <http://www.circresaha.org>. (*Circ Res.* 2002; 90:e40-e48.)

Key Words: cardiac tissue engineering ■ temperature-responsive cell culture surface ■ cell sheet
■ electrical communication ■ angiogenesis

Recently, cell transplantation to repair or supplement impaired heart tissue has been pursued by various approaches as an alternative therapy for heart transplantation.¹ Several possible cell types, including myoblasts, cardiomyogenic cells derived from bone marrow stroma, Lin[−]c-kit^{pos} bone marrow cells, multipotent endothelial cells, and embryonic stem cells, have been reported as human implantable cell sources.^{2–6} Myocardial injection of autologous myoblasts has been clinically performed and shown to produce some limited recovery from heart dysfunction.⁷ In these therapies using direct delivery of isolated cells, each cell differentiates and remodels in response to its surrounding environment, leading to tissue regeneration and functional repair.

In contrast to isolated cells, research on further advanced therapies to transplant tissue-engineered functional heart grafts has also now begun.⁸ As originally proposed by Langer and Vacanti,⁹ tissue engineering's most popular approach is based on the premise that preparations of cells, extracellular matrix (ECM), and growth factors together leads to tissue reconstruction, and that 3-dimensional (3-D) biodegradable scaffolds are useful as alternatives for ECM. This context has also been applied to cardiac tissue engineering. Eschenhagen, Zimmermann, and coworkers^{10,11} have developed a 3-D heart tissue model using a collagen matrix that allowed direct measurement of isometric contractile forces. Akins and coworkers¹² have shown 3-D contractile cardiomyocyte aggregates on polystyrene beads in rotating bioreactors. Papadaki

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and coworkers¹³ engineered 3-D cardiac constructs for in vitro impulse propagation studies using biodegradable polymer (PGA) scaffolds and rotating bioreactor. Cardiac grafts bioengineered using alginate scaffolds were reported to produce limited recovery from heart failure due to myocardial infarction.¹⁴ Furthermore, Li and coworkers^{15,16} have demonstrated that tissue-engineered cardiac graft transplantation using biodegradable gelatin mesh replaced both myocardial scar and right ventricular outflow track defects.

We now report our exploitation of a novel cell manipulation technique to construct 3-D tissues from culture by layering cell sheets without any biodegradable alternatives for ECM. To obtain viable cell sheets, cells are cultured on temperature-responsive culture surfaces. These surfaces are grafted with the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) by electron beam exposure, producing surfaces that are slightly hydrophobic and cell adhesive under culture condition at 37°C and change reversibly to hydrophilic and non-cell adhesive below 32°C due to rapid hydration and swelling of grafted PIPAAm.^{17,18} This unique surface change allows cultured cells to detach spontaneously from these grafted surfaces simply by reducing culture temperature. In contrast to enzymatic digestion (trypsinization), both cell-to-cell junctions and adhesive proteins within confluent cultured cell monolayers are perfectly preserved, resulting in production of intact cell sheets by detachment from PIPAAm-grafted surfaces.^{19,20} Various types of cell sheets have been successfully recovered and transferred to other surfaces.^{21–25} Presently, we have constructed 3-D tissues by layering cell sheets. Applications in cardiac tissue engineering require such tissue constructs to be electrically communicative and mechanically responsive in order to become functional heart tissue. Simultaneous beating of confluent cultured cardiomyocytes is preserved in vitro after confluent sheet detachment from PIPAAm-grafted surfaces.²⁴ Furthermore, we have demonstrated that electrical and morphological communications were established between double-layered chick embryonic cardiomyocyte sheets.²⁶ These results indicated the attractive potential of our technology for cardiac tissue engineering.

To extend this technology, the present study employs square-geometry neonatal rat cardiomyocyte sheets overlaid up to 4 layers in cultured tissue-like constructs. Tissue construct morphology and pulsatile characteristics of these cell-based graft constructs were analyzed in vitro and in vivo subcutaneous tissue.

Materials and Methods

All procedures using animals in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA.

Preparation of Square-Designed PIPAAm-Grafted Polystyrene Cell Culture Dishes

Specific procedures for preparation of square-designed PIPAAm-grafted cell culture dishes are described elsewhere.²² Briefly, IPAAm monomer (kindly provided by Kohjin, Tokyo, Japan) in 2-propanol solution was spread onto tissue culture polystyrene (TCPS) dishes (Falcon 3002, Becton Dickinson). Then, these dishes were then subjected to irradiation (0.25 MGy electron beam dose) using an Area Beam Electron Processing System (Nisshin High Voltage),

resulting in polymerization and covalent bonding of IPAAm to the dish surface. These PIPAAm-grafted dishes were rinsed with cold distilled water to remove ungrafted IPAAm and dried in nitrogen gas. In the second step, the PIPAAm-grafted surface was masked with a square glass coverslip (24×24 mm, Matsunami). Acrylamide (AAM) monomer solution in 2-propanol was spread onto the masked dish surface. Then the dish surface was irradiated with electron beam and washed. As a result, the center square area of each dish was PIPAAm-grafted (temperature-responsive) and surrounding border was poly-AAM-grafted (non-cell adhesive). Square-geometry PIPAAm-grafted dishes were gas-sterilized by ethylene oxide before use in culture.

Primary Culture of Neonatal Rat Ventricular Myocytes

Primary neonatal rat cardiomyocytes were prepared according to previously published procedures.²⁷ Briefly, ventricles from 1-day-old Wistar rats (Nisseizai, Tokyo, Japan) were digested at 37°C in Hanks' solution containing collagenase (class II, Worthington Biochemical). Isolated cells were suspended in the culture medium comprising 6% FBS, 40% Medium 199 (Gibco BRL), 0.2% penicillin-streptomycin solution, 2.7 mmol/L glucose, and 54% balanced salt solution containing (in mmol/L) 116 NaCl, 1.0 NaH₂PO₄, 0.8 MgSO₄, 1.18 KCl, 0.87 CaCl₂, and 26.2 NaHCO₃. The cell suspension was plated at a cell density of 8×10⁴/dish and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Manipulation of Cardiomyocyte Sheets Into Layered Constructs

Neonatal rat cardiomyocytes were cultured for 4 days at 37°C on thermoresponsive cell culture surfaces. To release confluent cells as a cell sheet, culture dishes were incubated in another CO₂ incubator set at 20°C. Cardiomyocyte sheets detached spontaneously within 1 hour and floated into the aqueous media. Immediately after detachment, the entire cell sheet with media was gently aspirated into the tip of a 10-mL pipet and transferred onto appropriate culture surfaces. Once placed, media is dropped onto the center of the sheet to spread folded parts of the transferred cardiomyocyte sheets. After sheet spreading, media was then aspirated to adhere the cell sheet to the culture surface. Within 30 minutes, the transferred sheet reattached and new media was then added for further culture. To layer cell sheets, another cardiomyocyte sheet detached from a PIPAAm-grafted dish was transferred into the first dish in the same way. The second sheet was positioned just above the first sheet and placed onto the original sheet by slow aspiration of media (30 minutes is sufficient for adhesion of layered sheets). Identical procedures were repeated to layer additional sheets. To construct pulsatile grafts in vitro, cardiomyocyte sheets were overlaid on frame-like collagen membranes (20-mm square, Cellgen, Koken), the center 5-mm square part of which was cut out to create an open frame into which the sheets were laid with center regions unsupported.

Cardiac Graft Transplantation Into Rats

Male F344 nude rats (6 3-week-old rats and 9 8-week-old rats; CLEA, Tokyo, Japan) were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg). The right dorsal skin was cut in a 3×2-cm angle and then opened. Cardiomyocyte sheets layered on PIPAAm-grafted surfaces were detached by lowering temperature in culture prior to surgery and then washed with Hanks' solution. The cell constructs were lifted on a polypropylene supporting sheet (2×1.5 cm) and transplanted onto dorsal subcutaneous tissues via sliding from the slippery polypropylene sheet. Skin incisions were then closed with 5-0 nylon sutures. At appropriate periods after these procedures, the rats were anesthetized as previously described. Electrograms of host hearts and transplanted grafts were recorded simultaneously. After that, transplanted areas were opened and a videotape recording was performed. The transplanted grafts were then resected for histological studies. The rats were then euthanized with overdoses of pentobarbital. Cell sheet bilayer constructs were transplanted into 3 3-week-old rats and 6 8-week-old

rats. Four-sheet layered cell constructs were transplanted into 3 3-week-old rats and 3 8-week-old rats. Three of the 8-week-old rats with bilayer constructs were examined at several periods (1, 8, or 12 weeks) after the transplantation. All other 12 rats were analyzed 3 weeks later.

Isolated Cell Injection

Eight 3-week-old male nude rats were injected with isolated cells in addition to the cell sheet transplantation. Specifically, 4 cardiomyocyte sheets were dissociated with trypsin and were suspended in 200 μ L serum-free media. All of these harvested cells (9×10^6 cells) were injected into left dorsal subcutaneous tissues through the skin using with a sterile 29-gauge needle. Four-sheet layered constructs were transplanted subsequently onto right dorsal subcutaneous tissue in the same animal and manner analogous to that described above. Histology and cell viability (TUNEL staining) were compared at 1, 2, 3, or 7 days after these procedures.

Microscopic and Macroscopic Observations of Cell Sheets

Still images of cultured cells and cardiac grafts were photographed by a digital camera (FinePix, Fujifilm). Size analysis of cultured cells was performed by measuring still images with NIH Image software. Microscopic images of detaching cardiomyocyte sheets and layered cardiomyocyte sheets were recorded by a digital video camera (DCR-TRV900, Sony) through a CCD camera (HV-D28S, Nikon). Macroscopic cardiac graft beating was also recorded by digital video camera. The systolic and diastolic long-axis lengths of the pulsatile transplanted tissue were measured by using still images imported into NIH image software. With systolic (S) and diastolic (D) values, fractional shortening was calculated as $D-S/D \times 100\%$.

Histological Analysis

For cell F-actin and nuclei staining, sheet samples were fixed with 4% paraformaldehyde. Cells were then stained with a 1:100 dilution of rhodamine-conjugated phalloidin (Molecular Probes, Eugene) for 2 hours and were costained with a 1:500 dilution of DNA-binding dye (Hoechst 33258) for 5 minutes. Plane and cross-sectional fluorescent images of the sheets were observed under a confocal laser scanning microscope (TCS, Leica). For cross-sectional observations, sheet-constructed grafts were fixed with formalin, embedded in paraffin, and sectioned into 10- μ m slices. Hematoxylin and eosin staining and Azan staining were performed by conventional methods. For actinin staining, samples were incubated with 1:500 diluted anti- α -sarcomeric actinin antibody (Sigma) over night and then with a 1:100 dilution of FITC-labeled anti-mouse IgG antibody (Wako). For vascular endothelial cell staining, samples were incubated with an anti-Factor VIII-related antigen antibody conjugated with horseradish peroxidase (Dako) overnight and then treated with chromogenic substrate solution for 5 minutes. To examine the presence of gap junctions, sheet constructs were frozen sectioned, fixed with 100% acetone, then incubated with a 1:100 dilution of anti-connexin43 antibody (Chemicon) for 3 hours. Then, samples were incubated with 1:200 diluted FITC-labeled anti-mouse IgG antibody. The images were photographed by a digital camera (Finepix S1 pro, Fujifilm) using fluorescence microscopy (ELIPSE TE300, Nikon). Thickness of the overlaid sheet constructs was measured by using micrograph images imported into NIH image software. To determine the quantity of connective tissue within transplanted cardiac tissue grafts, the blue area in Azan staining was measured by the same software. Total graft area was set at 100% and the ratio of blue area was calculated. Cardiomyocyte width was also measured at cell nuclei on hematoxylin and eosin-stained cross-section. The widths of 100 cells were measured and averaged for each cardiac graft.

TUNEL Staining

TUNEL staining of transplanted 4-layer constructs and injected cells was performed with an in situ DNA fragmentation detection kit (MK500, Takara). Frozen sections were fixed with 4% paraformaldehyde. Rat mammary gland was used as positive control. After

permeabilization, the samples were incubated with fluorescein-conjugated dUTP and TdT in a humidified chamber for 60 minutes at 37°C. Cell nuclei were counter-stained with a 1:500 dilution of Hoechst 33258. Fluorescence images were observed using the fluorescence microscopy.

Transmission Electron Microscopy

Transplanted cardiac grafts were fixed with 3% glutaraldehyde and postfixed with 1% osmium tetroxide. The samples were dehydrated by a graded ethanol series and embedded using Poly/Bed812 Embedding kit (PolyScience). Ultrathin sections were cut on an ultramicrotome (LKB-2088) and then stained with both uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (JEM-200EX, JEOL).

Electrophysiological Analysis

To detect electrical potentials of bilayer cardiomyocyte sheets separately, 2 cell sheets were layered by partial overlap. One week after the procedure, layered cell sheets were continuously superfused at 37°C with Tyrode's solution (containing in mmol/L: 137 NaCl, 3.7 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 5.6 glucose, and 4.0 Hepes) by using a heater controller (TC-344B, Warner Instrument) and flow pump (MasterFlex L/S Cole-Parmer Instrument). Two microelectrodes (100 μ m in diameter, Unique Medical) were positioned over a single part of each sheet. Electrical potentials were amplified by bioelectric amplifiers (UA102, Unique Medical) and were recorded by a data acquisition system (NR-2000, Keyence). Skin surface electrograms of nude rats were detected by using round plate electrodes (11 mm in diameter, Nihon Koden). For detection of host ECG, 3 electrodes were attached on right upper breast, left subcostal, and right femoral region. For cardiac graft electrograms, electrodes were positioned on transplanted site and bilateral femoral regions. Both electrograms were amplified and recorded simultaneously by the same equipment described above.

Force Measurement

A commercial Strain gauge (AE-801, SensoNor, width 0.95 mm) was impaled into the edge of 4-layer sheet grafts on the back of 3-week-old nude rats at 3 weeks after the transplantation ($n=3$). Contraction force was measured by force transducer (UPB-301, Unique Medical) connected to the gauge and recorded by the same data acquisition system described above.

Data Analysis

Data are expressed as mean \pm SD. An unpaired Student's *t* test was performed to compare 2 groups. One-way ANOVA was used for multiple group comparison. If the *F* distribution was significant, a Bonferroni's test was used to specify differences between groups. A value of $P < 0.05$ was considered significant.

Results

Detachment of Square-Geometry Neonatal Rat Cardiomyocyte Sheets

Neonatal rat cardiomyocytes were cultured on square-designed temperature responsive culture surfaces for 4 days and reached confluency. Adherent cardiomyocytes were 5.98 ± 0.12 cm² in area and 20 ± 4 μ m in thickness ($n=5$, Figure 1, top panels). The cells pulsed simultaneously and spontaneously at 88 ± 13 bpm ($n=6$). When culture temperature was decreased from 37°C to 20°C, cardiomyocytes detached as a contiguous cell sheet within 1 hour as shown in online Movie 1 in the online data supplement available at <http://www.circresaha.org>. No residual cells were observed on the culture surfaces after detachment. Released cardiomyocyte sheets shrank due to cytoskeletal tensile reorganization and were immediately transferred to TCPS surfaces by

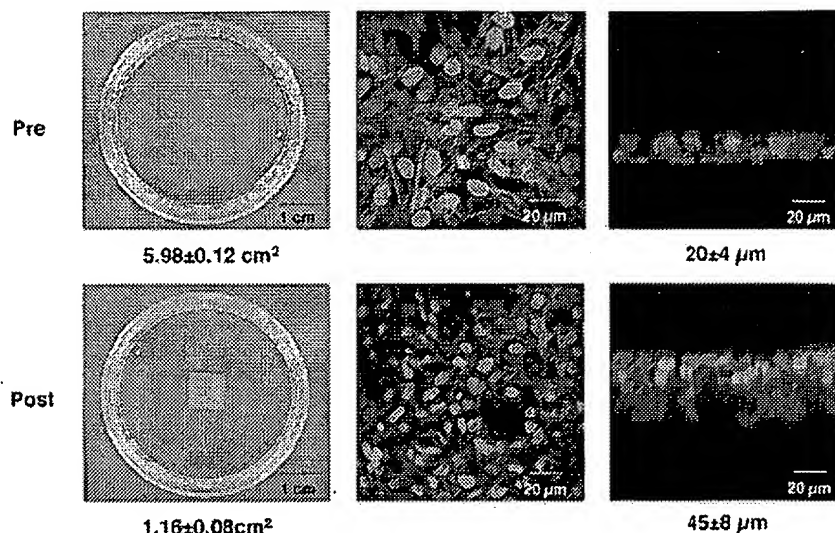


Figure 1. Cardiomyocyte sheets before (Pre) and after (Post) detachment. Neonatal rat cardiomyocytes cultured on a square-geometry PIPAAm-grafted dishes for 4 days (top panels) and recovered cardiomyocyte sheets after sheet detachment by reducing temperature (bottom panels) are demonstrated. Left panels show that the area of square cell sheets decreases from 5.98 ± 0.12 to 1.16 ± 0.08 cm². Center and right panels reveal images staining for F-actin (red) and cell nuclei (green). Spread cells are compacted after the detachment. In addition, sheet thickness increases from 20 ± 4 to 45 ± 8 μm in cross-sectional views over time free from support. Data are mean \pm SD; n=5.

pipette manipulation. The area of reattached sheets decreased to 1.16 ± 0.08 cm² and their thickness increased to 45 ± 8 μm (n=5, Figure 1, bottom panels). These shrunk sheets recovered spontaneous beating in a few days.

Electrical Communication Between Layered Sheets

To examine electrical communication between layered cardiomyocyte sheets, 2 cardiomyocyte sheets were layered partially on TCPS dishes as schematically illustrated in Figure 2A. Although beating of layered cell sheets initially stopped, overlaid sheets began to pulsate spontaneously and simultaneously within 1 week. Electrophysiological studies revealed that electrical spikes detected on the monolayer part of each sheet were completely synchronized at 74 ± 16 bpm (n=3) (Figure 2B). This result indicated that electrical communication was established between layered cardiomyocyte sheets.

Pulsatile Cardiac Graft In Vitro

As discussed in the previous article,²⁴ rigid culture dishes limit cardiomyocyte movements and release of cardiomyocyte sheets from culture surfaces augments pulsatile ability. Therefore, cardiomyocyte sheets were layered on 2-cm square frame-like collagen membranes, in which 0.5-cm square center parts were cut out. As a result, center parts of these bilayer constructs on frame-like collagen membranes were free from any culture materials, producing simultaneous waves in microscopic views as shown in online Movie 2. When 4 cardiomyocyte sheets were layered, macroscopic pulsation were observed (online Movie 3). Deformation of the collagen frame due to continuous beating was observed in a few days and spontaneous pulsation finally ceased. Cross-sectional views of bilayer sheets demonstrated fused homogeneous tissues and diffuse depositions of connexin43 within them (Figure 3). The center region thickness of bilayer and 4-layer constructs were 23 ± 3 μm and 53 ± 4 μm, respectively (n=3).

Surface Electrograms of Cardiac Grafts in Subcutaneous Tissues of Nude Rats

Layered cardiomyocyte sheets were transplanted into dorsal subcutaneous tissues of nude rats using a polypropylene sheet

as a transfer device (online Figure 1 in the online data supplement available at <http://www.circresaha.org>). At 3 weeks after the transplantations, surface electrograms originating from implanted cardiac grafts were detected independently from host electrocardiograms in 5 of 12 transplanted rats (online Figure 2). Graft beating rates varied from 13 to 96 bpm and were relatively slow in comparison with host hearts (332 ± 27 bpm, n=5). In the earliest case, the electrogram of the bilayer construct was detected at 2 weeks after the procedure.

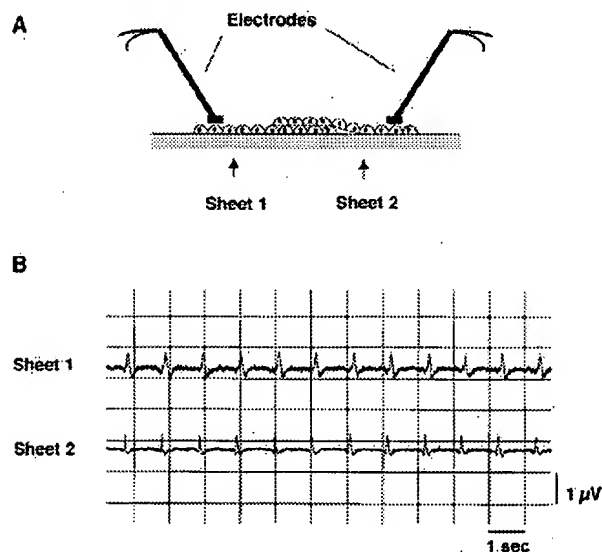


Figure 2. Electrical synchronization of layered cardiomyocyte sheets. A, Schematic illustration of electrical analysis of layered cardiomyocyte sheets is shown. Two cardiomyocyte sheets (Sheet 1 and 2) detached from PIPAAm-grafted culture dishes were transferred to a 60-mm dish and partially overlaid. Two electrodes were set over monolayer parts of both cell sheets and electrical potentials were amplified and detected. B, Representative tracings of detected electrical potentials of 2 sheets are shown. Electrical spikes detected on monolayer part of each sheet completely synchronized at 74 ± 16 bpm. Data are mean \pm SD; n=3.

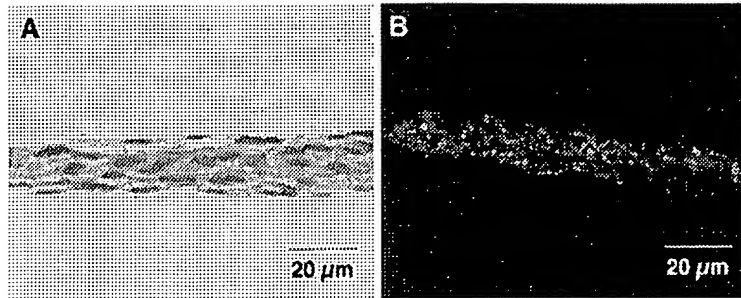


Figure 3. Cross-sectional views of bilayer cardiomyocyte sheets in vitro. Two cardiomyocyte sheets were layered on a frame-like collagen membrane. Cross-sectional views of free part of bilayer construct are demonstrated. A, Hematoxylin and eosin staining shows that bilayer sheets adhered intimately and became homogeneous tissue. B, Immunofluorescent study using anti-connexin43 antibody reveals diffuse gap junctions within the construct.

Macroscopic View and Functional Analysis of Transplanted Cardiac Grafts

When transplantation sites were opened at 3 weeks after transplantation, all transplanted grafts were positioned in the original transplanted areas and were pulsating spontaneously in macroscopic views ($n=15$). The macroscopic views of a bilayer construct resected with skin and a 4-layer graft left on dorsal muscle site were demonstrated in online Movies 4 and 5, respectively. Shapes of grafts transplanted in 8-week-old rats were nearly square, but a little distortion due to host growth was observed in the case of 3-week-old rats. Some vessels were observed growing into cardiac grafts, and microvascular nets within pulsatile grafts were observed. Fractional shortening values of 4 groups (bilayer constructs transplanted into 3-week-old rats, 4-layer into 3-week-old rats, bilayer into 8-week-old rats and 4-layer into 8-week-old rats, each $n=3$) were $6.7 \pm 0.6\%$, $8.9 \pm 0.5\%$, $3.6 \pm 1.4\%$, and $5.4 \pm 1.4\%$, respectively (Figure 4). These data demonstrated the tendency that fractional shortening increased depending on the number of layered cell sheets. Four-layer constructs transplanted into 3-week-old rats significantly shortened at higher ratios than those transplanted into 8-week-old rats. Contraction forces of 4-layer constructs transplanted into 3-week-old rats, measured in situ by a strain gauge, was 1.18 ± 0.26 mN ($n=3$). Macroscopic graft beatings were observed at the earliest period, 1 week after the transplantation. Pulsatile graft survival was also confirmed at least up to 12 weeks after the procedure.

Histological Analysis of Cardiac Grafts

Cross-sectional analysis of bilayer constructs transplanted into dorsal subcutaneous tissue of 3-week-old rats is shown in Figures 5A through 5E. Hematoxylin and eosin staining and azan staining revealed stratified cardiac tissue between connective tissues. No inflammatory changes were observed. Immunofluorescent staining of sarcomeric actinin showed well-differentiated sarcomeres of elongated cardiomyocytes. Multineovascularizations including red blood cells were detected in Azan staining and immunostaining with an anti-Factor VIII-related antigen antibody. Immunofluorescent images with anti-connexin43 antibody demonstrated diffuse formation of gap junctions in cardiac grafts. Transmission electron microscopic images demonstrated well-differentiated myofilaments, typical blood capillaries, and desmosomes within cardiac grafts (Figure 6). Cross sectional thickness of bilayer constructs transplanted into 3-week-old and 8-week-old rats were 41 ± 10 μm and 33 ± 10 μm , respectively ($n=3$). Transplanted 4-layer constructs were

65 ± 17 μm in 3-week-old rats and 69 ± 23 μm in 8-week-old rats ($n=3$). No significant difference in thickness due to host age was observed, but host age-dependent histological differences both in amount of connective tissue and in cell width were recognized (Figures 5B and 5F, 3-week-old and 8-week-old, respectively). The ratio of connective tissue within bilayer constructs transplanted into 3-week-old rats and 8-week-old rats were $32 \pm 3\%$ and $42 \pm 3\%$, respectively ($n=3$) (Figure 7A). The average graft cell width was 5.6 ± 0.4 μm in the case of 3-week-old hosts ($n=3$) and 3.8 ± 0.2 μm in 8-week-old hosts ($n=3$) (Figure 7B). These data demonstrate that the constructs transplanted into 3-week-old rats exhibited significantly more cardiomyocyte hypertrophy and less fibroblastic tissue within the grafts than those transplanted into 8-week-old rats.

Comparison Between Isolated Cell Injection and Cell Sheet Transplantation

To clarify the advantage of cell sheet transplantation over isolated cell injection, dissociated cells equivalent to 4 cardiomyocyte sheets were injected into left dorsal subcu-

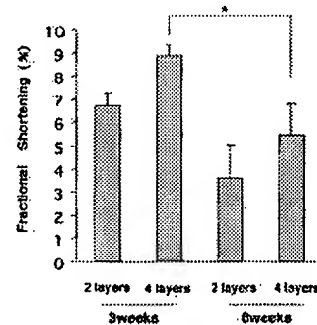


Figure 4. Fractional shortening of transplanted cardiac tissue grafts. Cardiac tissue graft beating was recorded by digital video camera (30 images/second) and both systolic (S) and diastolic (D) long-axis lengths were measured using still images imported into NIH image software. Fractional shortening was calculated as $D-S/D \times 100\%$. Fractional shortening of bilayer constructs transplanted into 3-week-old rats, 4-layer constructs transplanted into 3-week-old rats, bilayer constructs transplanted into 8-week-old rats, and 4-layer constructs transplanted into 8-week-old rats were $6.7 \pm 0.6\%$, $8.9 \pm 0.5\%$, $3.6 \pm 1.4\%$, and $5.4 \pm 1.4\%$, respectively (each $n=3$). Fractional shortening tended to increase depending on the number of layered cell sheets. Four-layer constructs transplanted into 3-week-old rats significantly shortened at higher ratios than those transplanted into 8-week-old rats. Values represent mean \pm SD. One-way ANOVA following Bonferroni's test was performed. * $P < 0.05$.

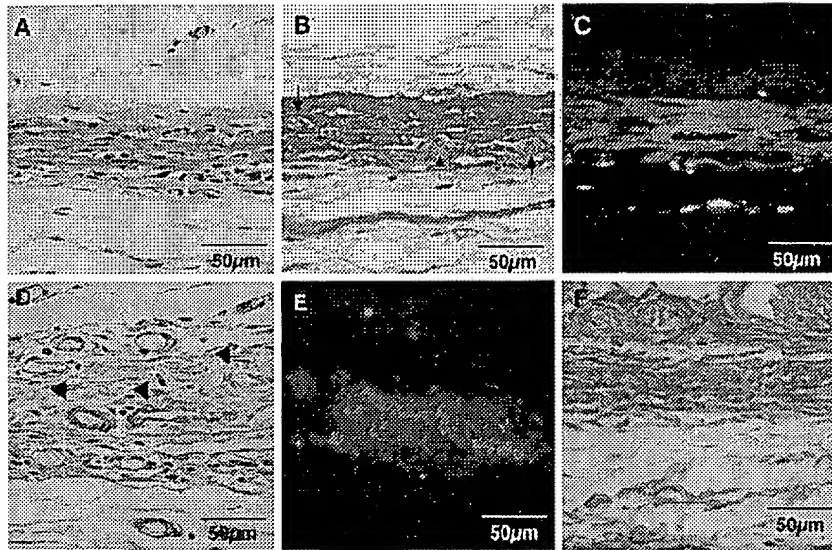


Figure 5. Histological analysis of cell sheet bilayer constructs transplanted into 3-week-old rats (A through E) or 8-week-old rat (F). A, Hematoxylin and eosin staining revealed stratified cardiac tissue within subcutaneous tissue. B, Cardiac tissue is clearly detected and micro vessels (arrow) including orange-stained red blood cells are detected in Azan staining. C, Immunofluorescent staining of sarcomeric actinin shows well-differentiated sarcomeres within elongated cardiomyocytes. D, Neovascularization within cardiac graft is also confirmed with endothelial cell-specific Factor VIII-related antigen staining (arrow head). E, Immunofluorescent image with anti-connexin43 antibody demonstrates diffuse formation of gap junctions in pulsatile cardiac grafts. F, Azan staining of cell sheet bilayer constructs transplanted into a 8-week-old rat. More connective tissue and less hypertrophic cardiomyocytes were observed within this construct than in construct transplanted into a 3-week-old rat (B).

taneous tissue and a 4-layer cardiac tissue construct was transplanted into right dorsal subcutaneous tissue. After the procedure, injected cells formed a bump under the skin, whereas cell-sheet-transplanted site was smooth. When both transplantation sites were opened 1 week later, an elliptic cell aggregate was observed at the left side and a flat square cardiac tissue construct was seen on the right side (online Figure 3A). Cross sectional view of the injected right-side construct showed cell-dense graft surface zones with cell-void central necrotic areas typical of high-density cell implants (Figure 8A). By contrast, the cell sheet construct exhibited homogeneous stratified tissue without observable necrosis (Figure 8E). Immunofluorescent images with anti-connexin43 antibody demonstrated that gap junctions had already established within the cell sheet construct in contrast to few depositions within the injected cells at 1 day after the operation (Figures 8B and 8F). TUNEL-positive cells were detected within the injected construct and most positive cells were observed at 2 days after grafting (Figures 8C and 8D). TUNEL-positive cells were hardly detected in the 4-layer cardiac tissue constructs at any periods (Figures 8G and 8H). Necrotic tissue including more eosinophilic cytoplasm, nuclei deformation, and neutrophils was also observed within the injected-cell graft (online Figure 3B).

Discussion

Cultured neonatal rat cardiomyocyte sheets were shown to be successfully layered, and electrical communications were established between the layered sheets. Four-sheet layered cell constructs pulsed macroscopically in vitro. Cardiac grafts transplanted into dorsal subcutaneous tissues revealed skin surface electrical potential, macroscopic beating, significant neovascularization, and heart tissue-like structures including sarcomeres, desmosomes, and gap junctions. Constructs transplanted into younger rats exhibited more cardiomyocyte hypertrophy and less connective tissue. These pulsatile cardiac grafts survived at least up to 3 months in subcutaneous implants in nude rats.

Cell sourcing remains a crucial problem for cell and engineered tissue transplantation. Various cell types have been examined as transplants into myocardial tissues.¹ Fetal cardiomyocytes and differentiated cardiomyocytes from embryonic stem cells have faced ethical problems. Autologous cells, including myoblasts and multipotent cells derived from bone marrow, may be the most desirable cell sources.^{4,7} With regard to delivery methods, dissociated cell transplants have been performed via direct myocardial injection, coronary artery injection, and an intraventricular approach. However, it has been reported that high levels of cardiomyocyte death occur at early periods after cell injection due to acute ischemia.²⁸ As shown in the present study, cells are more effectively delivered as a thin but large-area construct without cell loss using cell sheet transplantation in contrast to isolated cell injection with resulting central tissue necrosis. Furthermore, 2-dimensional shape and size are both controllable by grafting PIPAAm in appropriate designs on culture plates. Sheet construct thickness is regulated by the number of layered sheets desired. Connexin43 immunofluorescent image of the 1-day cell sheet constructs also demonstrated continuity of cell-to-cell communication preformed in vitro. These advantages in our cell delivery technology might result in improved efficiency of heart tissue repair.

As described in the Introduction, several reports have utilized 3-D biodegradable scaffolds as a transient support in cardiac tissue engineering.⁸ Insufficient cell migration into these scaffolds and inflammatory reactions due to scaffold biodegradation remain problematic.^{14,16} In contrast to this strategy, the present study demonstrates a new approach using viable intact cardiomyocyte sheets comprising cells with their own biologically produced ECM lacking any artificial scaffolds and layered 3-dimensionally to construct heart tissue. It is already well-known that confluent cultured cardiomyocytes express gap junctions and pulsate simultaneously.²⁹ These intimate electrical communications were preserved within cardiomyocyte sheets released from PIPAAm-grafted dishes on reducing culture temperature. To fabricate 3-D cardiac grafts, electrically communicative car-

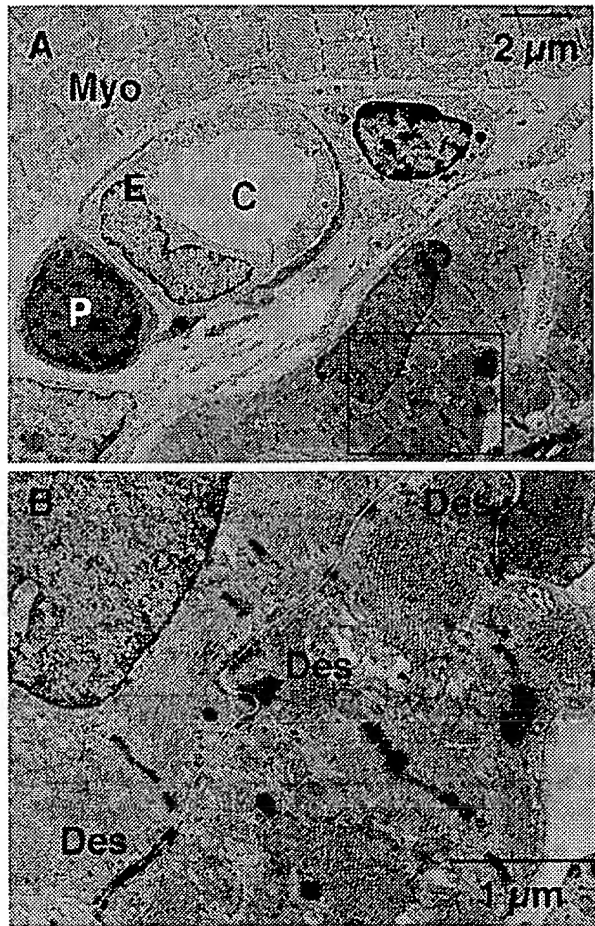


Figure 6. Transmission electron microscopic images of transplanted cardiac grafts. A, Well-differentiated myofilaments (Myo) are shown. Typical capillary (C) composed of several endothelial cells (E) and a pericyte (P) is observed within the construct. B, Desmosomes (Des), characteristic of connections of heart tissue, are present between cardiomyocytes.

diomyocyte sheets were layered into tissue-like multisheet constructs. Endogenous ECM preserved underneath each harvested cell sheet by the harvest method (eg, no trypsin) plays an important role as an adhesive agent.²⁰ Therefore, layered sheets adhere to each other, rapidly leading to 3-D tissue reorganization. As demonstrated both in previous and present studies using chicken and rat cardiomyocytes, electrical communications between layered sheets develop in culture, and the constructs beat simultaneously.²⁶ These data indicate that electrically connected thicker sheet constructs might be fabricated by layering additional cardiomyocyte sheets.

In this study, cardiac grafts were transplanted into subcutaneous tissues to examine pulsation and in vivo survival of implanted graft constructs. In order to more effectively restore heart dysfunction due to myocardial infarction, transplantation of layered cardiomyocyte sheets onto the myocardial scar may be beneficial. As discussed above, more cells should survive via cell sheet delivery over cell injection. In the treatment for dilated cardiomyopathy, for example, multiple cell injections are risky. Hence, placing cardiomyocyte

sheets onto wide areas of impaired heart tissue may prove a safer and more supportive therapeutic intervention. In either case, electrical and morphological communications between host hearts and grafts are important. Previously, tissue-engineered cardiac grafts using 3-D scaffolds exhibited acceptable tissue integration into the host by morphological analysis.^{14–16} These results encourage the application to damaged hearts of cardiac grafts fabricated by our technology. Novel physiological analysis may be needed to confirm host-graft electrical communication. Further studies are now ongoing in this context.

Sufficient perfusion of oxygenated blood is required for healthy beating heart tissue. The present study demonstrates multiple sites of neovascularization originating from subcutaneous tissue into the implanted cardiac grafts. This neovascularity may be induced by angiogenic factors including vascular endothelial growth factor secreted by cardiomyocytes.³⁰ Previous reports of cardiac tissue engineering utilizing 3-D biodegradable scaffolds have noted reconstruction with the graft periphery dense with cells, whereas the interior of the construct has necrotic or no cells due to insufficient oxygen perfusion.^{10,11,30a} Device-produced local hypoxia may also limit the maximal construct thickness fabricated by our technology without any preformed vascularity. As shown in the cross section of the injected cell constructs (Figure 8A), about 100 μm (about 6 neonatal rat cardiomyocyte sheets) may be the thickness limit for tissues in the absence of vascularization. To fabricate thicker viable tissue constructs, new techniques to control the density and directionality of newly grown blood vessels will be needed. Using our technology, a single layer of endothelial cells enhances the capillary formation in vivo.³¹ Therefore, deliberate insertion of endothelial cell sheets between cardiomyocyte sheets may promote neovascularization in situ, resulting in possibilities

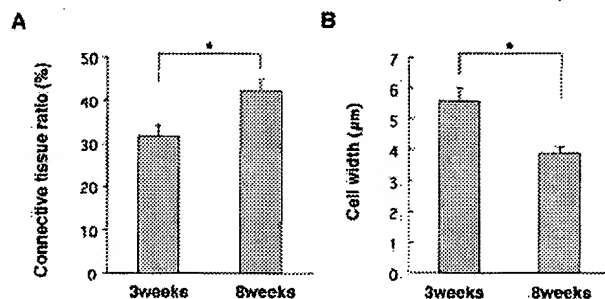


Figure 7. Host age-dependent histological differences of transplanted bilayer cardiac tissue grafts. A, In Azan staining, the ratio of blue area to the graft area (set at 100%) was measured by the NIH software to determine the quantity of connective tissue within transplanted cardiac tissue grafts. Bilayer constructs transplanted into 3-week-old rats includes significantly less connective tissue ($32 \pm 3\%$) ($n=3$) than those transplanted into 8-week-old rats ($42 \pm 3\%$) ($n=3$). B, Cardiomyocyte hypertrophy was examined by measuring cell width at the nucleus on hematoxylin and eosin-stained cross-sections. The widths of 100 cells were measured for each sample, and 3 samples were analyzed. The average graft cell width in the case of 3-week-old hosts ($5.6 \pm 0.4 \mu\text{m}$) was significantly larger than that of 8-week-old hosts ($3.8 \pm 0.2 \mu\text{m}$). Data are mean \pm SD. Unpaired *t* test was performed. * $P < 0.05$.

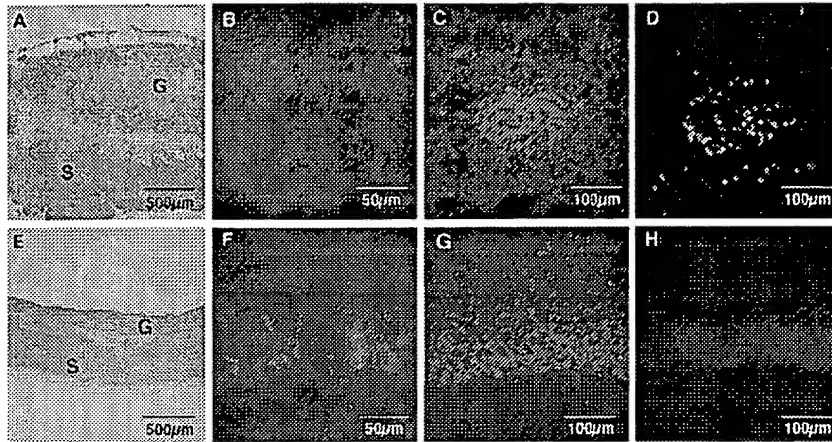


Figure 8. Histological comparison between dissociated-cell graft (A through D) and cell sheet graft (E through H). Cells dissociated with trypsin equivalent to 4 cardiomyocyte sheets were injected into left dorsal subcutaneous tissue, and subsequently an intact 4-layer construct was transplanted into right dorsal subcutaneous tissue contralaterally in the same animal. Hematoxylin and eosin staining of grafts (Gr) with dorsal skeletal muscle (S) fixed at 7 days are shown in A and E. Injected graft surface area (about 100 μ m) is cell-dense. However, the center region is hypocellular necrotic tissue (A). Cell sheet 4-layer grafts exhibit homogeneous stratified tissue without observable necrosis (E). Immunofluorescent images with anti-connexin43 antibody demonstrate that

well-organized gap junctions exist within the cell sheet construct at 1 day after the operation (F). By contrast, few gap junctions were observed within the injected cell sites (B). TUNEL staining of injected graft (D) and cell sheet graft (H) at 2 days after grafting are shown with nuclei counter staining (C and G, respectively). TUNEL-positive cells were detected within the injected construct. However, TUNEL-positive cells were hardly detected in the 4-layer cardiac tissue construct.

to create thicker tissue constructs. Further studies will be needed to clarify this crucial point.

Histological analysis of transplanted cardiac grafts in soft tissue demonstrated well-differentiated heart tissue-like structures in 3-week-old rats compared with 8-week-old rats. Functional analysis also showed 4-layer constructs transplanted into 3-week-old rats significantly shortened at higher ratios than those transplanted into 8-week-old rats. Host weight changes during transplantation periods were also quite different between 3-week-old rat procedure (from 64 ± 10 g to 151 ± 22 g) compared with the 8-week-old rat procedure (from 183 ± 10 g to 232 ± 23 g). Age-related fluctuations in growth hormones across these two ages, reported to correlate with myocardial development, may affect graft differentiation.³² As reported in several studies, mechanical stretch intensities may also cause differences in cardiomyocyte hypertrophic changes.^{33,34} These host age-dependent phenomena should be addressed further for future application of tissue-engineered constructs to adult patients.

Graft beating rates in subcutaneous tissues are relatively slow compared with host heart rates. This is proposed to originate in the isolation of cardiomyocytes from neonatal rat ventricles separate from atria, and lack of coordinated innervated stimuli. Another possible reason is the lower temperature of the dorsal subcutaneous tissue. In fact, a resected cardiac graft was observed to pulsate at a higher rate when media temperature was increased (data not shown).

The cardiomyocyte sheets, which readily detach from PIPAAm-grafted surfaces and transfer onto rigid culture surfaces or other cardiomyocyte sheets, stop their intrinsic beating temporarily but spontaneously recover within a few days. By contrast, when only one sheet is transferred onto frame-like collagen membranes, the center region begins to beat promptly after incubation at 37°C. Therefore, tissue reorganization between cell sheet-to-culture surface or cell sheet-to-cell sheet may cause the transient beating interruption, and it may take a few days for electrical transmission to recover within the constructs.

Layered cardiomyocyte sheets on frame-like collagen membranes pulsated as shown in online Movies 2 and 3. However, after several days, the floating collagen frame was bent due to continuous cell sheet contraction, resulting in construct shrinkage and aggregation. Then, sheet motion ceases. In vivo, the elastic connective tissue, which reacts against the graft contraction, was well-organized as shown in online Movies 4 and 5. Therefore, some elastic materials fixing the sheet constructs will be needed to facilitate continuously beating cardiac tissue grafts in vitro.

In conclusion, cell sheet manipulation technology (cell sheet engineering) utilizing temperature-responsive cell culture surfaces have been shown to be very useful for fabricating electrically communicative, pulsatile cardiac grafts both in vitro and in vivo. This technology should have enormous potential for constructing in vitro 3-D heart tissue models and improving viable functional cardiac graft materials for clinical tissue repair.

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